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Immunological Response in Cynomolgus Macaques to Porcine Alpha-1,3 Galactosyltransferase Knockout Viable Skin Xenotransplants – a Preclinical Study

Short Running Title: Porcine Xenotransplant Preclinical Study

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Abstract

Background

Allogeneic skin recovered from human deceased donors (HDD) has been a mainstay interim treatment for severe burns, but unfortunately risk of infectious disease and availability limitations exist. Genetically engineered alpha-1,3 galactosyltransferase knockout (GalT-KO) miniature swine used as source animals for viable skin xenotransplants may provide a promising clinical alternative.

Methods

Four cynomolgus macaque recipients received full-thickness surgical wounds to model the defects arising from excision of full thickness burn injury and were treated with biologically active skin xenotransplants derived from GalT-KO, Designated Pathogen Free (DPF) swine. Evaluations were conducted for safety, tolerability, and recipient immunological response.

Results

All skin xenotransplants demonstrated prolonged survival, vascularity, and persistent dermal adhesion until the study endpoint at postoperative day 30. No adverse outcomes were observed during the study. Varying levels of epidermolysis coincided with histological detection of CD4+ and CD8+ T cells, and other cellular infiltrates in the epidermis. Recipient sera IgM and IgG demonstrated significant antibody immune response to non-alpha-1,3-galactose porcine xenoantigens. Separately, specific wound healing mediators were quantified. Neither porcine cell migration nor PERV were detected in circulation or any vascularized organs.

Conclusions

These results provide a detailed analysis of vital skin xenotransplants utilizing a non-human primate model to predict the anticipated immunological response of human patients. The lack of adverse rejection even in the presence of elevated Ig indicates this is a prospective therapeutic option.

Keywords: skin xenotransplant, alpha-1,3 galactosyltransferase, GalT-KO, porcine endogenous retrovirus, porcine xenograft, rejection, human deceased donor allografts, pig

Introduction

Each year, burn injuries claim the lives of 180,000 victims worldwide ¹, and 100,000 Americans are hospitalized ². Young children are the most likely to experience these highly detrimental injuries ³. The ramifications of severe burns are profound for the patient, including long-term metabolic disturbances. When skin is significantly damaged, patients rapidly lose fluid, causing internal pH and homeostatic imbalances. If uncorrected, this leads to multi-organ failure in 50% of non-survivors of severe burns ⁴. Further, the sequelae of infection, sepsis, and inhalation injury combine to account for the death of three-quarters of all patients with injuries covering 40% or more of the total body surface area (TBSA) ².

Therapeutic options depend on the severity of the burn. Autografts are the ideal treatment as they create an effective barrier, cause minimal risk of infectious disease, and are immunologically compatible. However, autografts can be clinically contraindicated for patients with burns covering 20% or more TBSA ^{5,6}. In such scenarios, human deceased donor (HDD) allografts are an effective mainstay in the treatment of severe burns ⁷. HDD allografts are highly effective in creating a barrier for wound closure and thus in preventing infections. This involves formation of a fibrin seal which provides effective wound closure, and a critical bacteriostatic effect that is essential in the treatment of large burns ^{8,9}.

However, clinicians have long sought alternative treatment options that address the severe shortcomings of allograft material ^{7,10-12} while providing the same fundamental mechanism of action that achieves wound closure and temporary restoration of barrier function.

For decades, the field of xenotransplantation has represented a promising, but unrealized, solution to the global shortage of skin and other transplantable organs ¹³. Until the 1970's, classic xenografts derived from wild-type swine were used as temporary wound dressings, and are still used commonly today in some parts of the world ^{14,15}. However, these xenografts provide wound coverage – not wound closure – as preformed antibodies in humans to wild-type porcine skin led to antibody mediated, hyperacute rejection, considerably limiting adequate graft adherence to the wound and anastomosis with host vessels for meaningful durations ¹⁶. This hyperacute rejection is due principally to a unique antigen on porcine endothelial cells, the alpha-1,3-galactose (alpha-1,3-gal) epitope, which is recognized as foreign by preformed human antibodies.

Extensive research has provided a solution to this rapid rejection of porcine donor tissue in human recipients through genetic modification of source animals, made considerably easier with recently available technologies and gene editing techniques ^{17,18}. One of the most common and well-studied modifications is the removal of the alpha-1,3-gal epitope in genetically engineered alpha-1,3

galactosyltransferase knockout (GalT-KO) miniature swine¹⁹⁻²¹. As evidenced by preclinical studies, these GalT-KO skin xenotransplants delay the recipient's immune response^{22,23} and exhibit prolonged xenotransplant survival^{13,24,25}.

Immune rejection is not the only barrier to clinical xenotransplantation. Foremost, reduction of associated infectious disease concerns by using Designated Pathogen Free (DPF) donors will be essential to promulgating its large-scale use^{26,27}. However, to date there has not been in vivo evidence of PERV transmission in human patients exposed to pig cells or tissues^{26,32,33}. Long-term monitoring of potential PERV transmission and/or porcine cell microchimerism has also failed to indicate transmission of the virus from swine to human, even those cases followed for 34 years¹⁶.

The following preclinical study, performed under Good Laboratory Practices (GLP) conditions, was conducted in support of a U.S. Food and Drug Administration (FDA) Investigational New Drug (IND) application. Safety and efficacy were both primary considerations of the study, and the findings reported here directly supported regulatory clearance for a Phase I clinical trial.

In our first article describing this preclinical study³⁴, the primary focus was reporting the clinical observations that all xenotransplants demonstrated survival, adherence, and vascularity until postoperative day 30. Here, we present an extended analysis specific to the immunological response in recipients of vital skin xenotransplants, with a focus on the immune response to non-Gal porcine xenoantigens and presence of various immune mediators associated with wound healing.

Materials and Methods

Ethics

This study was conducted in accordance with U.S.-FDA GLP regulations and guidance documents (21 CFR Part 58.351), the U.S. Department of Agriculture's (USDA) Animal Welfare Act (9 CFR Parts 1, 2 and 3), the *Guide for the Care and Use of Laboratory Animals*³⁵ and the *Guidance for Industry documents: "Source Animal, Product, Preclinical and Clinical Issues for the Use of Xenotransplantation Products in Humans" and "Regulation of Intentionally Attended Genomics DNA in Animals"*. The study surgical procedures, protocols, and guidelines for animal care were independently reviewed and monitored by a standing institutional animal care and use committee (IACUC) committee. Lastly, the final study results were audited by an independent reviewer for quality assurance.

Xenotransplant Recipients

Two male, non-naïve and two female, naïve cynomolgus monkeys (*Macaca fascicularis*) of Chinese origin were assigned as graft recipients. This subject choice was necessary, as only humans and non-human primates possess preformed antibodies to the alpha-1,3-gal epitope, and thus reject wild-type porcine tissues in a similar manner^{36,37}. Furthermore, cynomolgus monkeys are well established in previous literature as scientifically appropriate subjects for such studies^{38,39}. Non-naïve animals were included as recipients in this study due to limited subject availability and to most ethically and prudently steward the use of research animals. These animals had previously been involved in pharmacokinetic studies that involved administration of drugs intended to reduce relapsed alcohol consumption and for treatment of cocaine abuse. Pharmacokinetic studies of this type were determined to have no known long-term immunological impact or likely interference with the experimental studies presented here. Pre-study physical examination by the testing facility's veterinary staff did not indicate any health issues that would preclude the animals from the study. Animal health, including clinical observations, body weight, body condition, food consumption, cardiac monitoring, respiratory rate, body temperature, neurological and pathological examinations were monitored/conducted at pre-determined, regular intervals throughout the duration of the experiment under veterinary supervision.

Procurement and Preparation of Skin Xenotransplants

Xenotransplants were obtained from source animals originating from a closed colony of DPF GalT-KO miniature swine originally developed by Sachs et al. at Massachusetts General Hospital (Boston, MA)

^{40,41}. The DPF designation signifies that the animals were raised under prescribed isolation conditions: raised from birth via Cesarean-section; confirmed porcine cytomegalovirus (PCMV) negative; hand-fed by gowned staff with sterilized food and water; housed separately in a Biological Safety Level 2 (BSL-2), positive pressure, temperature-controlled room with restricted access and no exposure to pigs outside of the housing area; vaccinated against normal swine pathogens and monitored on a quarterly basis for external swine pathogens to ensure the absence of several specific adventitious agents in line with the indicated guidance and ethics. One GalT-KO miniature swine from the closed colony was selected as the donor source animal for all eight skin xenotransplants used in this study. The preparation and procurement of the skin xenotransplants from the donor was previously described in detail ³⁴. The skin was procured under stringent aseptic conditions and sterile environment via air-driven Zimmer dermatome (Medfix Solution, Inc., Tucson, AZ). The harvested split-thickness porcine skin xenotransplants were inspected for quality, verified for thickness (0.55 mm/0.022 inches), and trimmed to form, measuring approximately 25 cm². This was followed by additional processing under sterile conditions, which carefully removed commensal skin flora and achieved United States Pharmacopeia (USP) <71> Sterility Standards ⁴² while retaining cellular viability. Skin xenotransplants were incubated in media containing a proprietary combination of antibiotics and antifungals, rinsed in saline, rolled in nylon mesh, and placed in a threaded-seal cryovial. CryoStor CS5 media, 5 mL, (BioLife Solutions, Bothwell, WA) was added to the vial, which was then sealed.

Vials were placed into a controlled-rate freezer, slow-cooled at a rate of 1°C per minute to -40°C, then rapidly cooled to -80°C before being transferred to a -80°C freezer. The cryopreservation method used in this study was evaluated in a previous study ⁴³, comparing identically prepared porcine skin grafts cryopreserved for different lengths of time. The results demonstrated no significant differences in clinical or histological outcomes, or in cellular viability as assessed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Combined, these specific practices retain the essential metabolic activity in porcine skin xenotransplants after cryopreservation and storage for extended durations.

The skin xenotransplants used in this study were cryopreserved for six months prior to thawing. Prior to surgery, skin xenotransplants were thawed by immersing the sealed vials in a 37°C water bath for approximately one minute, followed by three one-minute aseptic serial washes in sterile normal saline with gentle agitation. Thawed skin xenotransplants were then placed in sterile normal saline at ambient temperature and taken to the surgical field for use.

Surgical Transplantation of Xenotransplants

Transplantation surgery was performed on each of the four non-human primate (NHP) recipients via four sequential, independent surgical procedures. Animals were sedated with Ketamine (~15 mg/kg) and pre-medicated with Atropine (0.04 mg/kg IM). The animals were intubated and maintained on Isoflurane anesthesia (~1.0–2.5%, oxygen 2.0 liters). Prior to surgery, animals were given Buprenorphine (0.03 mg/kg IM) and Cefazolin (20 mg/kg IM). Hair was clipped from the dorsal and lateral thorax. The surgical site was prepared for surgery using three alternating scrubs of either povidone iodine or chlorhexidine scrub solution and sponges soaked in 70% isopropyl alcohol. The animals were moved to the operating table and positioned in sternal recumbency. Heart rate, respiratory rate, blood pressure (as applicable), end-tidal carbon dioxide, and body temperature were continually monitored throughout the procedure and recorded at least every 15–20 minutes as applicable. The surgical site was then prepared for aseptic surgery by wiping or spraying the surgical site with 70% isopropyl alcohol followed by an application of Dura-Prep™. A local anesthetic line block of 0.25–0.5 mL Lidocaine (or Bupivacaine) per site was performed prior to the procedure.

Wound beds were prepared in two stages, first by preparing partial-thickness wound beds using an air-driven Zimmer dermatome. These wound beds were then surgically converted into full-thickness wound beds using a scalpel, removing all tissue superficial to the underlying fascia. This resulted in two separate full-thickness 9 cm² wounds on the dorsal aspect of each subject, between the inferior aspect of the scapulae and superior to the iliac crests, centered medially along the spine. Split-thickness skin xenotransplants were trimmed to fit the wound bed and uniformly sutured in place, covering the entire wound, using simple, interrupted, 3-0 nylon sutures. In total, eight (n=8) skin xenotransplants were placed across the four subjects (n=4).

Postoperative Care and Wound Dressing of Recipient Subjects

Postoperative care for the recipients was described in detail by Holzer et al ³⁴. Briefly, all wounds and overlying skin xenotransplants were covered with pressure dressings, VetRap and primate jackets. Animals received Buprenorphine (0.03 mg/kg, IM) immediately on postoperative day 0 (POD-0) and every other day thereon as required. Clinical observations were performed at least twice daily. At no time during this study was immunosuppressive therapy administered to the transplant recipients.

Gross assessment of the wound sites occurred on postoperative days 7, 14, 21, and 30 (end of study). During each observation, primate jackets and dressings were removed, the wounds were cleaned, the

wound sites were evaluated for duration of survivability (i.e., time to immune-mediated rejection) of the skin xenotransplants, and were redressed as previously described. At each dressing change, peripheral blood was obtained for later evaluation. At the conclusion of the study, all xenotransplant recipients were euthanized and a complete necropsy was performed.

Postoperative Histopathological Assessment

Approximately 5 mm³ pieces of major tissues (the spleen, liver, kidney, lung and heart) were obtained from each animal at necropsy. These major tissues and wound sites were collected and fixed in 10% neutral buffered formalin (NBF). The major tissues were then transferred to 70% ethanol after approximately 72 hours (\pm 4 hours). Wound sites, major tissues and additional organs were shipped to Alizée Pathology (Thurmont, MD) for histological processing and evaluation. On receipt, wound sites were trimmed, processed, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E).

Immunohistochemistry was performed on the wound sites to look for the presence of CD4+ and CD8+ T cells. Paraffin-embedded tissues were cut at 4 μ m thickness, mounted on glass slides and incubated at 60°C for 30 minutes, followed by deparaffinization in xylene and rehydration in graded alcohol into water then tris-buffered saline (TBS)/Tween. Antigen retrieval was performed by Diva Decloaker (Biocare, CA) for 15 minutes. Endogenous blocking was done with normal goat serum and avidin for 20 minutes. The tissues were incubated with 1:20 dilution of CD4 mouse monoclonal antibody (Biocare ACI3148A Clone 4B12) or 1:20 dilution of CD8 mouse monoclonal antibody (Biocare ACI3160A, Clone CD8/144B) in van Gogh diluent (Biocare, CA) at 4°C overnight. After washing, slides were incubated via the avidin-biotin complex method for 20 minutes⁴⁴. This was followed by 4+ Biotinylated Universal Goat Link Polymer horse radish peroxidase, (Biocare, Agilent, CA) probe and polymer each for 20 minutes at room temperature. After washing, DAB (3,3'-diaminobenzidine) reagent (DAKO; Agilent, CA) was added with monitoring for 5 minutes. Counterstaining was performed using Harris hematoxylin. Slides were briefly dehydrated and then mounted with Cytoseal Mounting Media (Life Technologies, NY).

Postoperative Assessment of Recipient Immune Response

Peripheral blood (PB) was obtained for isolation of serum and peripheral blood mononuclear cells (PBMCs) on POD-0, 7, 14, 21, and 30. Isolated serum was aliquoted and placed into a -80°C freezer until analyzed. PBMCs were harvested from PB samples by lysing the red blood cells in RBC lysing solution

twice and then washing in phosphate-buffered saline. The resulting PBMCs were pelleted, lysed, and frozen at -80°C until use.

To evaluate the inflammatory and anti-inflammatory wound healing cytokines and chemokines, characteristic of initial wound healing processes, recipient sera was analyzed using a Luminex 23-plex assay, Milliplex Map Non-Human Primate Cytokine Magnetic Bead Panel (Millipore Sigma, PRCYTOMAG-40K, Merck KGaA, Darmstadt, Germany). The multiplexed assay panel was specific for cross-reactivity with NHP samples. The panel of 23 mediators measured in this assay included the following: interleukin-1ra (IL-1ra), IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12/23(p40), IL-13, IL-15, IL-17, IL-18, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 alpha (MIP-1 α), macrophage inflammatory protein-1-beta (MIP-1 β), soluble CD40 ligand (sCD40L), transforming growth factor-alpha (TGF- α), transforming growth factor-beta (TGF- β), vascular endothelial growth factor (VEGF), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF) and interferon-gamma (IFN-gamma).

In addition, binding of recipient serum IgM and IgG antibodies to non-Gal porcine xenoantigens in the skin xenotransplants was measured by flow cytometry. PBMCs from GalT-KO swine were isolated from the buffy coat of porcine peripheral blood and counted using a Coulter MD II Hematology Analyzer. Viability was measured using a Countess II FL Automated Cell Counter (Life Technologies). Serum samples were de-complemented in a 56°C dry heat bath for 30 minutes and serially diluted at 1:2, 1:10, 100, 1,000, and 10,000 in Flow Activated Cell Sorting Media (FACS) (1X Hanks Balanced Salt Solution with Ca²⁺ and Mg²⁺, 0.1% Bovine Serum Albumin (BSA), and 0.1% Sodium Azide). 10 μ L of serially diluted serum samples were incubated with 1.5×10^5 cells in 100 mL FACS buffer for 30 minutes at 4°C. Cells were washed twice and stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgM and phycoerythrin-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 30 min at 4°C. Cells were washed twice with FACS buffer and data acquisition was performed with an ACEA NovoCyte Flow Cytometer. Appropriate compensation, Limit of Blank (LOB) (which is the mean fluorescent intensity of cells using only secondary antibody in the absence of serum), and Fluorescence Minus One (FMO) controls were run. Binding of IgM and IgG was assessed using mean fluorescence intensity (MFI) and relative MFI values which were obtained as follows: Relative MFI = Actual MFI value / LOB.

Postoperative Assessment of Porcine Endogenous Retrovirus (PERV)

Samples of liver, spleen, kidney, lung, residual skin xenotransplant, and underlying wound bed were obtained at necropsy. These, along with lysed PBMCs and sera obtained concomitant with each clinical assessment, were dedicated for assessment of postoperative transmission of PERV. DNA was isolated from the PBMCs and wound beds using the Gentra Puregene kit (Qiagen, Crawley, UK). RNA was isolated from tissue samples using the RNeasy mini kit (Qiagen, Crawley, UK). Viral RNA was isolated from serum using the Viral RNA mini kit (Qiagen, Crawley, UK). DNA PCR assays for PERV and microchimerism (porcine centromeric DNA) were performed as previously described¹⁶. For viral RNA, each reaction was spiked with Taqman exogenous internal positive control (Applied Biosystems, UK). For total RNA, reactions included 18S RNA as a reference using probe and primers from the 18S rRNA control kit (Eurogentec, Hampshire, UK). This reference was used to avoid false negatives due to the absence of RT-PCR failures and/or PERV RNA.

Statistical Analysis

Simple mean and standard deviation were used exclusively. A student t-test was used to compare changes in cytokine/chemokine levels to baseline and a paired t-test was used to compare the change in IgG and IgM binding from POD-0 to POD-30.

Results

Postoperative Survival and Tolerability Assessment

All xenotransplant recipients tolerated the initial surgical procedure and placement of bilateral skin xenotransplants without issue. All four subjects survived to the scheduled end of study, POD-30, without experiencing any adverse events. Each recipient lost weight following the surgical procedure, but maintained a body condition score ranging between 2.5 (lean) and 3 (optimum). Percent reduction in body weight for each recipient, between preoperative evaluation and the end of study (POD-30), was -3.8% (subject 2001), -8.2% (subject 2002), -10.0% (subject 2101), and -9.4% (subject 2102). These values are within acceptable ranges following a surgical event of this nature.

Postoperative Clinical Assessment

As previously reported³⁴, all eight skin xenotransplants adhered completely to the underlying wound bed; zero technical failures were observed. Over the course of the study, each xenotransplant independently demonstrated prolonged survivability and vascularity relative to previous studies that utilized similar models and study designs^{24,45-47}, based on the color, texture, capillary refill, and overall clinical appearance.

At no time was sloughing of the xenotransplant clinically visible or exposure of the underlying wound bed observed. Epidermolysis (mild to moderate) was first noted on POD-14, but the dermis at all eight sites remained completely adherent. By POD-21, progression to complete epidermolysis was noted. Later assessments also revealed wound bed granulation and signs of re-epithelialization, such that by POD-30, varying degrees of re-epithelialization (up to 100% in some cases) had occurred in all four subjects (Figure 1/Table 1).

Postoperative Histopathological Assessment

There were no systemic histopathological differences observed between POD-0 and POD-30, following a comprehensive examination which included assessment of the external body and all orifices, as well as the cranial, thoracic, abdominal cavities, and their contents. Histological examination of kidney, lung, liver, spleen and heart samples demonstrated no evidence of toxicity related to the xenotransplants.

As previously reported ³⁴, hematoxylin and eosin (H&E)-prepared sections of skin xenotransplants and wound beds obtained at the end of study were microscopically evaluated by a blinded pathologist. H&E staining showed minimal to moderate inflammatory response. Ulceration of the epithelia was observed in four out of eight treated sites. The wound sites were initially viewed at a lower magnification to identify the border of the host tissue and remaining tissue of the skin xenotransplant (Figure 2). Wound defects were characterized histopathologically by the presence of a mature dermal collagen network distinct in appearance from the host dermis bordering the wound site, interpreted to be dermis of the xenotransplant, surrounded by a variable layer of new collagen (Figure 2). Features of dermal fibrosis, focal thinning of the epidermis and granulation tissue were also noted. Edema was minimal and considered within normal range. No additional staining was performed to confirm this.

A second pathologist further assessed the skin xenotransplants for cellular infiltrations and other microscopic indicators of immunological rejection via two methods ⁴⁸. The Banff Classification for skin-containing composite tissue allograft pathology was used to categorize xenotransplant rejection. A component scoring system designed to complement the Banff system was also used to identify variations in cellular infiltration and other pathologic lesions. These results are shown in Table 1. The Banff Grades ⁴⁹, based on the level of epidermal apoptosis, epidermal infiltrates, and perivascular/dermal infiltrates, ranged from II (epidermal infiltration) to IV (necrotizing acute rejection), with most showing Grade III (severe). Multiple foci of perivascular inflammation and epidermal infiltrates with apoptosis were observed. Cellular infiltrates included macrophages, lymphocytes, neutrophils, eosinophils, and plasma cells. CD4+ and CD8+ T cells were identified in perivascular and epidermal infiltrates (Figure 3). Multinucleated giant cells associated with granulomatous inflammation were also present (not shown).

Postoperative Assessment of Recipient Immune Response

Twelve of the 23 cytokines/chemokines assayed were consistently below the level of detection throughout the entire study period: TNF- α , IFN- γ , TGF- β , G-CSF, GM-CSF, IL-1- β , IL-4, IL-5, IL-10, IL-13, IL-17, IL-18, and MIP-1- α . VEGF exceeded the level of detection at only 3 of 20 individual time points, and levels of MIP-1-beta were discernable only once (data not presented).

Nine mediators detected over the period of the study were observed to increase above background at POD-7, the first day of sampling, and are listed in Table 2. IL-2, IL-8, MCP-1 and TGF- α peaked at

POD-7 and decreased over time. IL-15 and IL-12/23 (p40) peaked at POD-14, while sCD40L, IL-1ra and IL-6 had an elevated peak at POD-21. In general, all detectable mediators showed a return to normal by POD-30 with the exception of sCD40L, which remained elevated at POD-30. Of interest, levels of IL-12/23 (p40) were nearly absent until conspicuously elevated on POD-14, gradually reducing in concentration over the remainder of the study.

In addition, the binding of recipient serum anti-porcine IgM and IgG to porcine non-gal xenoantigens from GalT-KO donors was assessed by flow cytometry. Serum IgM and IgG antibody levels from each recipient, at POD-0 and POD-30, were analyzed by flow cytometry. In Table 3, the relative mean fluorescent intensity (MFI) and fold increase in binding are summarized for each recipient. An increase in anti-porcine IgM and IgG antibodies was detected in all animals. From levels measured pre-transplant to those detected at the end of study, IgM anti-porcine antibodies increased between 1.4 to 4.9 fold ($p=0.0095$), and IgG anti-porcine antibodies increased between 28.7 to 70.8 fold ($p=0.0003$).

Postoperative Assessment of Systemic Porcine Cell Migration and PERV

Naïve skin xenotransplants from the DPF donor were analyzed for PERV copy number. Each cell contained copies of PERV A (32 ± 1), B (9 ± 0.1) and C (16 ± 0.1). Post-xenotransplant, the presence of PERV was found in four of eight wound beds (3/4 recipients) at the site of the xenotransplants. This is likely due to localized porcine cell migration, as evidenced by the positive results from the microchimerism assay (Table 4).

Regardless of the limitations of the animal model for PERV infection, due to evidence of porcine cellular presence, PBMC samples from each of the four recipients were tested for microchimerism (i.e., the presence of circulating pig cells) and for PERV. All samples tested negative. Sera from the four recipients were also evaluated for the presence of circulating PERV; all samples were found to be negative for PERV *pol* and below the limit of detection. Liver, spleen, kidney, and lung tissues taken at the end of the study (POD-30) were evaluated for PERV expression and were also found to be negative.

Discussion

30-Day Survival of Xenotransplant Exceeds Previous Findings in Similar Models

The skin xenotransplants in this study demonstrated adherence, vascularity, and restoration of barrier function beyond previous published findings, and well beyond those same characteristics demonstrated by acellular, non-vital porcine xenografts, such as aldehyde cross-linked porcine dermis⁵⁰. Prior reports using earlier versions of GalT-KO materials^{25,46} reported an average of 10-14 day graft survival, while true wild-type, GalT+ skin xenotransplants exhibited a clinical appearance of “white grafts” as early as POD-4^{24,45-47}, an indication of ischemic injury caused by preformed antibodies against porcine endothelial cell alpha-1,3-galactose epitope²⁴. In contrast, all eight cryopreserved and vital (i.e., possessing metabolically active cells) skin xenotransplants evaluated in the present study, sourced from GalT-KO miniature swine from a DPF closed colony, remained visibly adherent at POD-30 without the administration of immunosuppressive agents. These are encouraging findings with promising clinical implications.

The exact cause for the observed prolonged xenotransplant survival is a topic of continued investigation. We hypothesize that the use of DPF source animals, particularly those negative for PCMV and other various adventitious agents, and the stringent pre- and post-operative conditions of a third party facility required to meet regulatory compliance, likely had a combined, advantageous impact on the ultimate survival of the skin xenotransplants. PCMV positive porcine organs into non-human primates has been reported to significantly reduce porcine xenotransplant survival time^{28,51,52}, but it has not been reported for xeno skin transplants. The donor animals used in this study were routinely screened and known to be PCMV-negative. In addition, use of meticulous aseptic procurement and sterile processing methods, which achieved USP<71> levels of sterility for the xenotransplants, are novel and have not been previously reported. Combined with optimized freeze-thaw protocols and maximum retention of post-thaw viable, metabolically active cells, it is possible that collectively these too had a positive effect on the survival of the skin xenotransplants.

In contrast to our preceding study³⁴ – wherein skin xenotransplants were shown to perform similarly to skin allotransplants, without discernable impact on subsequent autotransplants – in this study, only xenogeneic materials were employed. In order to isolate the observed immunological effects to those only related to the xenotransplants by avoiding unnecessary confounding factors or introduction of potential “bystander” effects, neither allogeneic nor autologous materials were used. Further, the four recipients in the present study were naïve subjects, as discussed, and had not previously received a xenotransplant.

Lastly, it could be posited that these findings could be attributed to the existence of subtle or nuanced variations of the immunological response to non-Gal, porcine xenoantigens between species. In the majority of previous, similar studies^{24,25,45,46}, baboon recipients were used instead of cynomolgus macaques. However, Fujita et al. used a cynomolgus monkey model and showed a survival time (nine days) comparable to what was reported in the baboon model⁵³. It is generally agreed among investigators that the most valid outcome comparisons should be assessed within the same model system.

The key clinical observation from the present study was that the xenotransplant dermis remained adherent to the wound bed until POD-30, and full sloughing of the graft was not reported as in previous studies⁴⁵⁻⁴⁷. Histological assessment of xenotransplant rejection using Banff grades ranged from II (epidermal infiltration) to IV (total epidermal necrosis). In the regions of epidermal necrotization, cellular infiltrates included CD4+ and CD8+ T cells.

Postoperative Anti-Porcine Antibodies and Cytokine Concentrations

For xenotransplantation to be successful, both humoral and cell-mediated immune responses must be adequately mitigated in order to prevent rejection of the foreign transplanted organ. In the present study, measurement of anti-porcine IgM and IgG antibodies specific to non-Gal porcine xenoantigens from GalT-KO donors demonstrated a significant increase in the humoral response to non-Gal porcine antigens over the 30 day study period.

In a similar model using GalT-KO miniature swine donors and baboon recipients, Albritton, et al.⁴⁵ described an acute onset of anti-porcine IgM antibodies with subsequent decline, later followed by an observable increase in IgG antibodies. The use of GalT-KO donor swine eliminates alpha-1,3-gal as the target antigen, and several additional non-Gal porcine xenoantigens have been identified which are likely the focus of the IgG antibody response^{16,54,55}. Additional non-gal antigens such as Neu5GC or SLA were not investigated in this study. Further, it has been demonstrated that these non-Gal xenoantigens can be eliminated with additional genetic knockouts introduced to donor source animals^{54,55}, rendering xenotransplants derived from such donors immunologically more similar to allotransplants.

Examination of wound healing cytokines and chemokines, characteristic of initial wound healing processes, provided unique insights into underlying immune mechanisms caused as a result of our xenotransplants. Many of the anticipated proinflammatory cytokines prevalent acutely following the

precipitating trauma or injury event were not detectable at POD-7, and may have resolved below the level of detection by the first postoperative evaluation ⁵⁶. Circulating levels of sCD40L were elevated at POD-7 and remained elevated to the end of study. This observation is consistent with those in surgical trauma patients. Activated platelets release sCD40L, a mediator that links inflammation, hemostasis and vascular dysfunction, and is associated with tissue/endothelial damage and platelet activation ⁵⁷. It also plays an important role in inflammation by increasing the expression of cell adhesion molecules, cytokines, and matrix metalloproteinase-1 (MMP-1), all of which are associated with neovascularization ⁵⁷. Given the criticality of adequate perfusion of the skin transplant for survival after the initial imbibition phase, the finding of elevated sCD40L levels present at the end of the study is in line with the clinical healing observed at each wound site.

Other wound healing factors such as TGF- α were detected with a peak at POD-7. TGF- α is a mitogenic polypeptide that is a ligand for epidermal growth factor receptors and can stimulate the proliferation and development of epidermal cells. It is produced by platelets, activated macrophages, and keratinocytes at the wound site. Previously, topical administration of TGF- α in a porcine wound model was shown to improve wound healing of the damaged skin ⁵⁸. It has also been shown to stimulate the migration of keratinocytes to the wound site for initiation of repair and has been detected in the serum at the wound site ⁵⁹. Similarly, IL-8, a chemotactic factor for neutrophils ⁶⁰, and MCP-1, a chemotactic factor for monocytes/macrophages ⁶¹ were also elevated during the inflammatory response of the wound and repair process, at POD-7.

Elevated serum levels of IL-12/23(p40), which is involved with wound repair ⁶² and is produced by activated inflammatory cells such as macrophages, neutrophils, dendritic cells, and keratinocytes ⁶³, was notably detected at POD-14. IL-12/23(p40) is composed of two cytokines, IL-12 and IL-23, which have distinct roles associated with CD4+ naïve T cells. The IL-12 portion promotes differentiation to TH₁ effector cells that stimulate natural killer cells and CD8+ T cell production of IFN- γ . The IL-23 portion stimulates generation of TH₁₇ cells, shown to be beneficial in wound repair ⁶⁴. Wound repair studies in IL-12/23(p40) knockout mice demonstrated that IL-12 and IL-23 modulate early inflammatory responses and subsequent angiogenesis ⁶². The transient peak at POD-14 of IL-12/IL-23(p40) is possibly the result of an overproduction during wound healing. Lastly, IL-23 is reported to be associated with regulating the expression of IL-17 ⁶⁵, shown to be essential in driving the macrophage population from proinflammatory to pro-repair. Although the results of our study did not detect any IL-17 in serum, it could be postulated that IL-17 was locally confined to, or consumed at, the wound site.

No Evidence of Systemic Porcine Cell Migration or PERV Transmission Observed in Xenotransplant Recipients

While PERV was present in four of eight wound beds at the site of the xenotransplants, the positive results from the microchimerism assay are likely an indicator of localized porcine cell migration, resulting from the intimate contact between the porcine xenotransplant and the perfused wound bed.

No evidence of PERV DNA or RNA was detected in samples of sera, PBMC, spleen, liver, lung or kidney tissues evaluated from the four subjects at the time of sacrifice. Overall, these results demonstrate that porcine DNA and systemic porcine cell migration into peripheral circulation of the recipients could not be detected. Altogether, these results provided no evidence of PERV transmission, consistent with previous studies^{16,26,32,33,66,67}.

It could be argued that a main caveat of this portion of the present study, with respect to PERV transmission, is the limitation of utilizing NHPs as models of PERV infection. It is well known that the lack of receptors for PERV cause NHPs to be the best available, but not ideal, animal model for such evaluation⁶⁸. No preferred alternatives exist.

To be clear, while the animal model used in this study did not provide evidence of a lack of PERV transmission, it is prudent to evaluate tools to be used in a clinical trial. FDA guidance states that recipient screening is required in clinical applications⁶⁹. Although to date, there is still no evidence of PERV transmission to human recipients of porcine xenotransplants or circulatory migration of porcine cells^{26,70-74}, we believe that our assessment of porcine cell microchimerism and absence of PERV detection presented here are relevant and satisfy the relevant regulatory guidelines required. While not evaluated here, a follow-up beyond 30 days would have been informative.

Applicability of Findings to Clinical Evaluation of Skin Xenotransplants

The complexity of the immune reaction of a patient with severe burns must be taken into account when designing a study intended to evaluate safety and tolerability of xenotransplants for clinical use. The design of the present study was intended to create an in vivo model that could adequately consider, in a predictive capacity, risk factors associated with the xenotransplant combined with those of the injury itself.

A considerable body of existing clinical and nonclinical data demonstrate that such vital, GalT-KO porcine skin xenotransplants offer a promising alternative option to HDD allotransplants for temporary wound coverage in severe and extensive, deep partial- and full-thickness burn injuries^{24,45-47}.

This study supports these previous findings and provides further evidence that xenotransplantation represents a safe and efficacious clinical alternative. Clinical efficacy combined with the elimination of risks from deceased donor-related disease transmission through the use of DPF source animal donors, as well as scalability and increased material availability would provide a significant therapeutic benefits in patients with severe burn injuries.

Due in part to these findings, the U.S.-FDA granted clearance to proceed with a Phase I evaluation of safety and tolerability of a vital porcine xenotransplant to provide temporary wound closure of severe and extensive, deep partial- and full-thickness burn wounds. Patient enrollment for a first-in-man xenotransplantation trial commenced in 2019, at Massachusetts General Hospital, Boston, MA.

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Table 1. Banff Grades and Pathologic Component Scores† of Skin Xenotransplants at POD-30

Animal	Graft	Surgeon assessment	Banff Grade	pc‡	pa§	ei¶	e††	v‡‡	c§§	cav¶¶
2001	1	100% re- epithelialized	III	3	3	3	1	0	1	0
2001	2	100% re- epithelialized	III-IV	3	3	3	2	0	1	0
2002	1	30% re- epithelialized	III-IV	3	3	3	3	0	0	0
2002	2	30% re- epithelialized	III-IV	3	3	3	3	0	0	0
2101	1	40% re- epithelialized	II	3	3	2	3	0	0	0
2101	2	40% re- epithelialized	III-IV	3	3	3	2	0	0	0
2102	1	20% re- epithelialized	III-IV	3	3	3	3	0	0	0
2102	2	20% re- epithelialized	II-III	3	3	3	3	0	0	0

† Pathologic Component Scores developed by Rosales, et al. ⁴⁸

‡ pc = perivascular cells – number of cells surrounding dermal vessels (venules, capillaries, and arterioles) in deep and superficial dermis; scored on the most involved vessels; pc3 >50 cells/vessel

§ pa = perivascular dermal infiltrate area –percent area occupied by the most involved dermal vessels at 40x magnification; pa3 >75%

¶ ei = epidermal infiltrate – total number of mononuclear cells per four 20x fields; ei3 = transepidermal infiltrate, ei2 >20 cells

†† e = epidermal injury and necrosis – presence of keratinocyte apoptosis and necrosis; e3 = sloughed, e2 = focal necrosis, e1 = apoptosis

‡‡ v = endarteritis – mononuclear cells underneath arterial endothelium; scored on the most involved artery; v0 = none

§§ c = capillaritis – maximum number of cells per capillary cross section; scored on most involved capillaries; c1 = 2–4/capillary, c0 = 0–1/capillary

¶¶ cav = chronic allograft vasculopathy – intimal thickening with luminal reduction; scored as percent luminal reduction; cav0 = none

Table 2. Changes in Serum Cytokines and Chemokines after Xenograft Transplantation (pg/mL)					
Cytokine/ Chemokine	POD-0	POD-7	POD-14	POD-21	POD-30
sCD40L	1900 ±1000	7900† ±3100	7700†‡ ±3100	8600†‡ ±4000	8500†‡ ±5200
IL-1ra	7.6 ±2.8	50† ±44	28† ±11	66† ±83	24† ±13
IL-2	29 ±11	42† ±18	37† ±11	41† ±9	30 ±12
IL-6	0.31 ±0.6	7.3† ±8.3	4.1† ±2.6	8.5† ±6.3	3.3† ±2.7
IL-8	2500 ±1300	4200† ±3200	3700† ±2600	3900† ±2300	2500 ±2100
IL-12/23 (p40)	0.6 ±1.0	1.8 ±2.7	26† ±22	16† ±11	6.7† ±7.7
IL-15	3.1 ±1.9	6.0† ±2.0	7.1† ±1.3	5.0† ±1.3	6.0† ±1.5
MCP-1	360 ±150	710† ±540	420† ±110	460† ±110	310 ±120
TGF-α	4.5 ±4.6	22† ±11	16† ±11	5.2 ±3.6	9.9 ±8.9

POD = Postoperative day

Values are means (n=4) ± SD

† Significant datapoints (p<0.05) compared to POD 0, student t-test

‡ Values include data at the upper level of detection (12,000 pg/mL)

Table 3. Post-Transplant Changes in Binding of Recipient Serum IgM and IgG to PBMC† Targets

from GalT-KO[‡] Swine Donors

Recipient	Pre/Post-Transplant [§]	IgM ^{††}		IgG ^{‡‡}	
		rMFI [¶]	Fold Change	rMFI [¶]	Fold Change
2001	Pre	8.51	0.0	16.28	0.0
	Post	45.72	4.4	1089.85	65.9
2002	Pre	5.07	0.0	28.29	0.0
	Post	30.01	4.9	840.64	28.7
2101	Pre	7.92	0.0	16.03	0.0
	Post	22.48	1.8	730.83	44.6
2102	Pre	6.47	0.0	5.19	0.0
	Post	15.49	1.4	372.88	70.8

[†] PBMC= peripheral blood mononuclear cell

[‡] GalT-KO= alpha-1,3 galactosyltransferase knockout

[§] Pre-transplant=POD-0; Post-transplant=POD-30

[¶] rMFI = relative Mean Fluorescent Intensity

^{††} IgM = immunoglobulin M

^{‡‡} IgG = immunoglobulin G

Table 4. Data for Postoperative Analysis of Wound Beds (Wound Site 1 and 2)				
Animal ID	Wound Site	PERV copies/500ng (SD)	Micro- chimerism[†]	QC[‡]
2001	W1	<LOD [†]	-	+
	W2	1495.6 (±521)	+	+
2002	W1	1518.8(±21)	+	+
	W2	<LOD	-	+
2101	W1	527.1 (±134)	+	+
	W2	137.8 (±16)	+	+
2102	W1	<LOD	-	+
	W2	<LOD	-	+

SD = Standard Deviation, LOD = Limit of Detection, QC= Quality Control

[†] Porcine microchimerism cannot be accurately quantified due to mixture of cells present in wound bed extraction

[‡] All QC gave a positive Ct, indicating no inhibition

Figure Legends

Figure 1. Skin xenotransplants on dorsal surface of Animal 2001 at POD-30

- A. Low power image of wound site.
- B. High power image of wound site.

Figure 2. Wound site 1, Animal 2002 at POD-30

- A. 40x power image of wound site. There is incomplete epithelial coverage (region of ulceration) indicated by double-headed arrow. Open arrowheads indicate new collagen surrounding residual xenograft.
- B. 200x power image of large inset box in Figure 2A. Above the dotted line is a region of new collagen with prominent inflammatory infiltrate attributed to region of ulceration at the surface. Below the dotted line most of the tissue consists of xenograft tissue with some inflammation (arrowheads), and some new collagen (open arrows).
- C. 200x power image of small inset box in Figure 2A. To the left of the dotted line at the arrow is native host dermis, while residual xenograft dermis lies to the right of the line (open arrows). Note the difference in collagen morphology (black arrow = host dermal collagen; open arrow = xenograft dermal collagen).

Figure 3. Wound bed, Animal 2001 at POD-30

Histologic sections reveal multiple foci of perivascular inflammation and epidermal infiltrates with apoptosis (Banff Grade III). Features of dermal fibrosis, focal thinning of the epidermis and granulation tissue were also noted (A, B). CD4+ and CD8+ cells (200x) were present in perivascular and epidermal infiltrates, as well as eosinophils, some neutrophils and plasma cells (C, D).

- A. 40x H&E staining of wound bed containing epidermal infiltrates into the xenotransplant at POD-30
- B. 200x H&E staining of wound bed containing epidermal infiltrates into the xenotransplant at POD-30
- C. 200x epidermal infiltrates stained for CD4+ cells in the xenotransplant at POD-30
- D. 200x epidermal infiltrates stained for CD8+ cells in the xenotransplant at POD-30